

A TRIBASIC STAIN FOR THIN SECTIONS OF PLASTIC-EMBEDDED, OsO_4 -FIXED TISSUES

PHILIP M. GRIMLEY, *Pathologic Anatomy Department, National Cancer Institute, National Institutes of Health, Bethesda 14, Maryland*

ABSTRACT. Thin ($0.5\text{--}1\ \mu$) sections of plastic-embedded, OsO_4 -fixed tissues were attached to glass slides by heating to $70\text{ }^\circ\text{C}$ for 1 min. A saturated solution combining toluidine blue and malachite green was prepared in ethanol (8% of each dye) or water (4% of each dye). Methacrylate or epoxy sections were stained in the ethanol solution for 2–5 min. The water solution was more effective for some epoxy sections (10–30 min). Epoxy sections could be mordanted by 2% KMnO_4 in acetone (1 min) before use of the aqueous dye, reducing staining time to 5–10 min and improving contrast. Aqueous basic fuchsin (4%) was used as the counterstain in all cases; staining time varied from 1–30 min depending upon the embedding medium and desired effects, methacrylate sections requiring the least time. In the completed stain, nuclei were blue to violet; erythrocytes and mitochondria, green; collagen and elastic tissue, magenta; and mucin and cartilage, bright cherry red. Sections were coated with an acrylic resin spray and examined or photographed with an oil-immersion lens.

Buffered osmium tetroxide provides excellent fixation of fine structure and has been applied generally in electron microscopy (Porter and Kallman 1953). More recently it has been used in conjunction with glutaraldehyde fixation (Sabatini *et al.* 1963). Unfortunately, its use as a fixative in light microscopy has been limited by the basophilic staining reaction of the fixed tissue. Such basophilia prevents differentiation of nuclear and cytoplasmic components by routine histologic methods (Baker 1958). Use of a simple combination of basic dyes which could be applied without removal of the embedding medium in methacrylate or epoxy thin-sections was therefore investigated. In general, highly concentrated dye solutions were required to obtain optimal intensity of staining in thin ($0.5\text{--}1\ \mu$) sections. Several basic dyes of the phenyl methane and quinone-imine series provided satisfactory results. These were individually evaluated with regard to staining affinity for various cell and tissue components. Saturated ethanolic solutions of several basic dyes including toluidine blue, malachite green, crystal violet and auramine O provided stains which could be counterstained with aqueous basic fuchsin. A combination of toluidine blue and malachite green was chosen as the basis for a "tribasic" procedure, since these dyes produced complementary tinctorial effects and could also be used in aqueous solution.

MATERIALS AND METHODS

Material from normal rats, including myocardium, kidney, intestine, pancreas, liver, pituitary, and blood were used. These were prepared as for electron microscopy by standard techniques (Pease 1960). Fixative solutions consisted of OsO_4 , buffered according to Caulfield (1957) or Millonig (1961), or glutaraldehyde followed by OsO_4 (Sabatini *et al.* 1963). Castings in Araldite 502 (Ciba) or Epon 812 (Shell) were prepared according to the recommendations of Luft

(1961). Thin sections were obtained with Porter-Blum microtomes and diamond (Du Pont) or glass knives. Sections were lifted from the floatant with a finely shaven wooden applicator stick and refloated on a drop of distilled water at one end of a glass slide. To eliminate mechanical wrinkling, the sections were spread with xylene (Satir and Peachey 1958) before and after transfer. The water drop was permitted to evaporate at room temperature. Sections were then fixed by heating to 70 C on a hotplate for 1 min. For staining, slides were allowed to stand in Coplin jars 1–30 min.

A saturated dye solution was prepared with equal parts by weight of toluidine blue¹ and malachite green², in either absolute ethanol (8% of each dye) or distilled water (4% of each dye). The solutions were stored in sealed Coplin jars. Surface film was removed from the aqueous solution at necessary intervals by gently wiping with absorbent tissue. The ethanolic dye solution acted more rapidly and did not develop a surface film so that it was preferred for all methacrylate castings and some epoxy castings (particularly Araldite). Sections were immersed for 2–5 min until cell nuclei were well stained. Some epoxy resin (particularly Epon) occasionally stained diffusely in the ethanolic dye solution. When such staining of embedding matrix was objectionable, the aqueous dye solution was used, with a staining time of 10–30 min. Epoxy sections could be mordanted in a 2% acetone solution of KMnO_4 for 1 min, prior to the application of the aqueous dye solution; staining time was then reduced to 5–10 min and contrast was improved. Excess dye was washed from the slides by gentle flooding with tap water.

A saturated (4%) aqueous solution of basic fuchsin¹ served as a counterstain and differentiator. Before use, excess dye was permitted to settle from the solution in order to prevent accumulation of particles on the immersed slides. Staining time in this solution had to be varied according to the embedding medium, section thickness, and degree of differentiation desired. Methacrylate sections required only 1–3 min for effective counterstaining and differentiation, and were easily overstained. Epoxy sections generally required more than 5 min and occasionally as long as 30 min to achieve desired results. Certain structures (*e.g.* collagen) counterstained rapidly, while other components (*e.g.* brush borders) required longer for differentiation.

After rinsing with water, the sections were dried and coated with an acrylic resin³ to retard fading. This resin, originally recommended as a substitute for cover slips (Vroman 1953) was sprayed lightly over the thoroughly dried sections, allowed to dry, and successive applications made until a coat of desired thickness was obtained. Since the spray solution slowly dissolved methacrylate, particular caution was exercised to spray light coats and allow complete drying between applications on methacrylate sections. Coated sections were examined

¹ Toluidine blue O, Cert No. NU-14, and basic fuchsin, Cert No. NF-76 were obtained from National Aniline Division of Allied Chemical Corp., New York 6, New York.

² Malachite green, Cert No. EMg-3 was obtained from Fisher Scientific Co., Fairlawn, New Jersey.

³ Krylon, crystal clear, No. 1303, Krylon Inc., Norristown, Pennsylvania.

to best advantage with an oil-immersion objective, since the low refractive index of acrylic resin (1.49) produced optical aberration at high-dry powers.

RESULTS AND COMMENTS

Ethanol solutions of the basic phenylmethane dye derivatives generally showed a stronger affinity for osmiophilic structures (*e.g.* erythrocytes and mitochondria) than the counterpart aqueous solutions which stained collagen, elastic tissue and certain granules (blood neutrophil, pituitary basophil) more intensely. This provided a basis for the use of aqueous basic fuchsin as a counterstain to ethanol solutions of other basic dyes. A more versatile combination was made possible by the fact that malachite green retained a strong affinity for osmiophilic structures even in aqueous solution and was thus complemented by toluidine blue which stained nuclei well in either ethanol or water solutions. Sections stained in a combination of these dyes were readily counterstained or differentiated with aqueous basic fuchsin. In the case of elastic tissue, collagen and certain granules, basic fuchsin appeared to act as a true counterstain, since these structures failed to stain or stained only weakly in the primary solution.

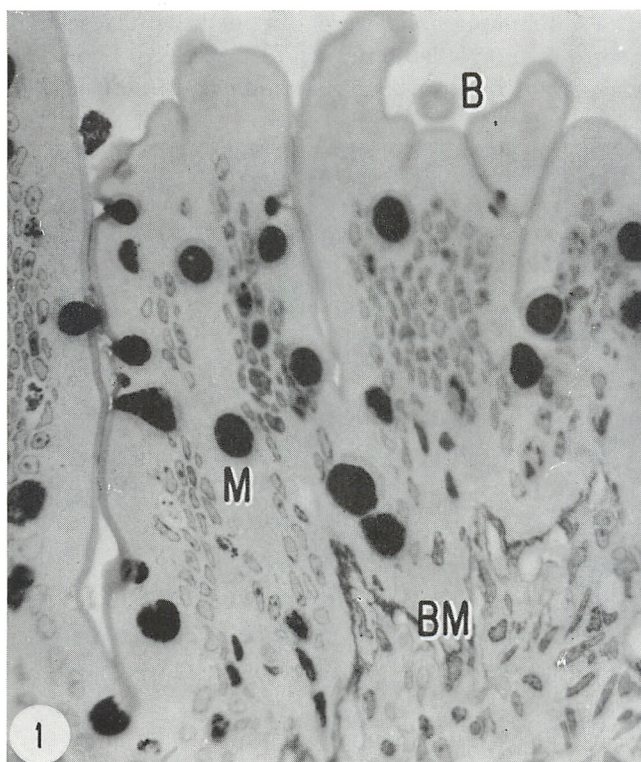


FIG. 1. Rat intestine, $0.75\ \mu$ section, Araldite embedding, OsO_4 fixation, "tribasic" stain. Mucin droplets (M) bright red; nuclei, blue; basement membrane (BM) and associated collagen, magenta; brush border (B), pink. $\times 630$.

In the case of ectoplasmic components (epithelial microvilli, brush borders, minor processes of renal podocytes) and some granules (pituitary basophil, pancreatic zymogen) the initial green or blue stain was selectively replaced or superimposed upon by basic fuchsin, producing various shades of magenta. This differentiation was time related, and overexposure to the basic fuchsin resulted in a diffuse nonspecific replacement. In the correctly timed stain, mitochondria, erythrocytes, and certain granules (blood eosinophil) remained green. Lipid droplets were green, but retained a grayish hue due to the presence of OsO_4 reduction products. The metachromatic effects of toluidine blue were masked by parallel staining effects of the malachite green or basic fuchsin. Mucin and cartilage were nevertheless distinguished by a bright cherry red color and mast cell granules were a bright aqua blue in the completed tribasic procedure. The use of KMnO_4 as a mordant was empirical and did not significantly alter the described color patterns.

The trichrome effects described showed much more contrast of cytoplasm, nuclei and connective tissue components than that seen in toluidine blue stains now widely used (Bencosme *et al.* 1959, Trump *et al.* 1961). Sections of $0.5\text{--}0.75\ \mu$ provided optimal clarity and intensity with the saturated dye solutions recommended. Photomicrographs were obtained within several days, since fading appeared to be unavoidable in such thin sections. Figure 1 demonstrates the clear contrast of mucin droplets in a section of rat intestine. Figure 2 demon-

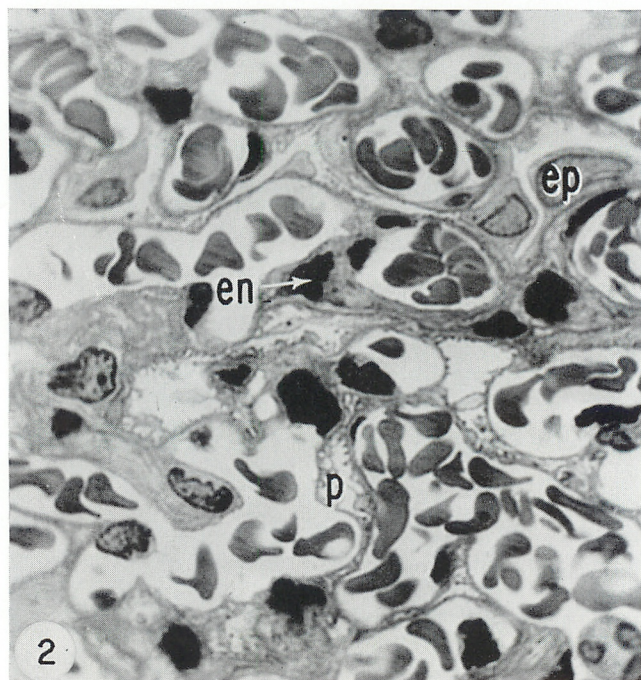


FIG. 2. Portion of rat renal glomerulus, $0.5\ \mu$ thick, same processing as in Fig. 1. Erythrocytes green; endothelial cell nuclei (en), bluish violet; epithelial cell nuclei (ep), blue; nucleoli, dark blue; basement membrane, magenta; foot processes (p), pink. $\times 1,600$.

strates the fine details of glomerular structure which can be revealed in a photomicrograph. Of particular interest is the deeper intensity of toluidine blue stain acquired by endothelial cell nuclei, in contrast to the paler epithelial cell nuclei, thus facilitating identification of these cells.

Fixation by the precise methods evolved for electron microscopy combined with plastic embedding yielded a preservation of morphology for light microscopy unobtainable by conventional techniques, hence exquisite detail was shown after staining (*cf.* Flax and Caulfield 1962). The staining by three basic dyes embodies a principle similar to that involved in the more familiar usage of three acid dyes ("triacid" stains); namely that the various tissue components will stain selectively if the dyes are suitably chosen. This selectivity is apparently governed not only by dye structure, but relative dye concentration, staining time and rate of penetration through the embedding medium. Thus simple mixture of the three dyes in a single solution did not produce desired results. The "tribasic" stain described is suitable for routine use, class demonstrations and photomicrography.

ACKNOWLEDGMENTS

This work was supported in part by USPH Training Grant 2G-349 in the Department of Pathology, University of California Hospital, San Francisco, California. Grateful acknowledgment is made to Dr. Marilyn G. Farquhar for use of laboratory facilities and materials, as well as helpful advice. Thanks are due to Mrs. Karin Taylor for technical assistance, and to Dr. Wilfred Torreson for use of photographic facilities.

REFERENCES

- BAKER, J. R. 1958. Principles of Biological Microtechnique. Methuen and Co., Ltd., London.
- BENGOSME, S. A., STONE, R. S., LATTA, H., and MADDEN, S. C. 1959. A rapid method for localization of tissue structures or lesions for electron microscopy. *J. Biophys. Biochem. Cytol.*, **5**, 508-9.
- CAULFIELD, J. B. 1957. Effects of varying the vehicle for osmium tetroxide in tissue fixation. *J. Biophys. Biochem. Cytol.*, **3**, 827-9.
- FLAX, M. H., and CAULFIELD, J. B. 1962. Use of methacrylate embedding in light microscopy. *Arch. Path.*, **74**, 387-95.
- LUFT, J. H. 1961. Improvement in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.*, **9**, 409-14.
- MILLONIG, G. 1961. Advantages of a phosphate buffer for OsO_4 solutions in fixation. *J. Appl. Phys.*, **32**, 1637.
- PEASE, D. C. 1960. Histologic techniques for electron microscopy. Academic Press, New York.
- PORTER, K. R., and KALLMAN, F. 1953. The properties and effects of osmium tetroxide as a tissue fixative with special reference to its use for electron microscopy. *Exp. Cell Res.*, **4**, 127-9.
- SABATINI, D. D., BENSCH, K., and BARNETT, R. J. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.*, **17**, 19-58.
- SATIR, P. C., and PEACHEY, L. D. 1958. Thin sections. II. A simple method for reducing compression artifacts. *J. Biophys. Biochem. Cytol.*, **4**, 345-7.
- TRUMP, B. F., SMUCKLER, E. A., and BENDITT, E. P. 1961. A method for staining epoxy sections for light microscopy. *J. Ultrastruc. Res.*, **5**, 343-8.
- VROMAN, L. 1953. Acrylic spray as a substitute for coverslips. *Am. J. Clin. Path.*, **23**, 516.
- 398

